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This fellowship was initially awarded to Dr. Wesley Hung who was a postdoctoral fellow in Dr. Bruce Elliott's laboratory (1998-2000). After Dr. Hung's departure, a new trainee, Dr. Ewa Joanna Wojcik, was recruited to the group as an MSc graduate student in Pathology. Approval to transfer the remaining funds of the award to support Dr. Wojcik from July 1, 2002 to June 30, 2003 was granted by the USAMRMC (see attached letter). Dr. Elliott continues his supervisory role of this project. In addition, Dr. Christopher Mueller, who has extensive expertise in regulation of gene transcription and promoter analysis, functions as co-supervisor. This report provides a brief update of the current aims and preliminary results of the revised program. A more detailed report will be provided at the end of the award period.

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Preface

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Introduction

Background Information

Hepatocyte Growth Factor (HGF) is a multifunctional cytokine, and its expression is required for normal breast development.(8) However, it is normally expressed only by stromal cells, while epithelial cells express HGF receptor- Met.(1) In contrast to what is observed in normal epithelium, HGF and Met are frequently overexpressed in breast carcinomas, and this high expression has been described to be an independent predictor of poor survival in patients with breast cancer.(3,9,11) These results suggest that establishment of an autocrine HGF loop and sustained activation of the Met signal transduction pathway in carcinoma cells may promote tumor progression. However, the mechanisms leading to aberrant expression of HGF in carcinoma cells are not known.

A number of signalling molecules such as c-Src, Grb2/Ras and PI3-kinase have been shown to be part of the HGF/Met signalling pathway.(2,6,7) One of them, c-Src is constitutively activated in a mouse breast carcinoma cell line, SP1, which expresses HGF and Met; and its activity is required for HGF-dependent cell motility and anchorage independent growth of these cells. It is also known that overexpression of an activated form of c-Src in transgenic mice induces mammary hyperplasia.(10) These findings indicate that c-Src kinase is an important requirement, but is not sufficient for mammary tumourigenesis.

Activation of c-Src kinase can lead to increased expression of many genes, including growth factors such as vascular endothelial growth factor (VEGF), (5) and c-Src inhibition by treatment with PP2 was observed to cause a 2-fold reduction in *HGF* transcription in SP1 cells.(4)

Using deletion mutants of the *HGF* promoter, a region (between –254 and –70) was located, which is responsive to increased c-Src activity in SP1 cells. Two putative consensus binding sites for Stat3 were located in this region. The role of Stat3 in c-Src dependent regulation of *HGF* transcription was examined, and a strong cooperative activation of HGF transcription was observed after simultaneous expression of Stat3 and activated c-Src in both, malignant and non-malignant breast epithelial cells.(4)

These data suggest that c-Src kinase and Stat3 act cooperatively in the activation of HGF expression in breast carcinoma cells, and may be important in overriding the strong repression of HGF expression in non-malignant epithelial cells.

Hypothesis

Based on the above discussion we hypothesize that Stat3 binding sites, located in the region between –254 and –70 of the *HGF* promoter, are required for the cooperative activation of *HGF* transcription by c-Src and Stat3, and this activation plays an important role in overriding the strong repression of HGF expression observed in normal breast epithelial cells. This process may lead to the establishment of an autocrine HGF loop and sustained activation of the Met signal transduction pathway in carcinoma cells, which can promote tumor progression.

Objectives

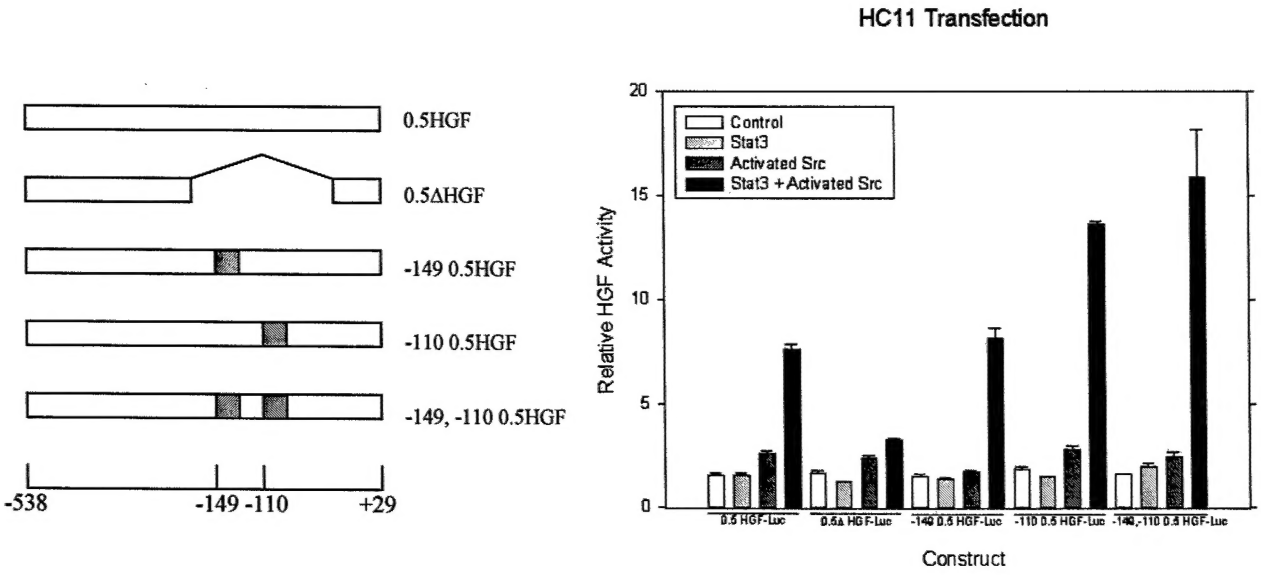
1. Identification of the site responsible for c-Src/Stat3 responsiveness of *HGF* promoter.
2. Testing of activated Stat3 function in *HGF* expression
3. Characterization of DNA-protein complex formation

Body

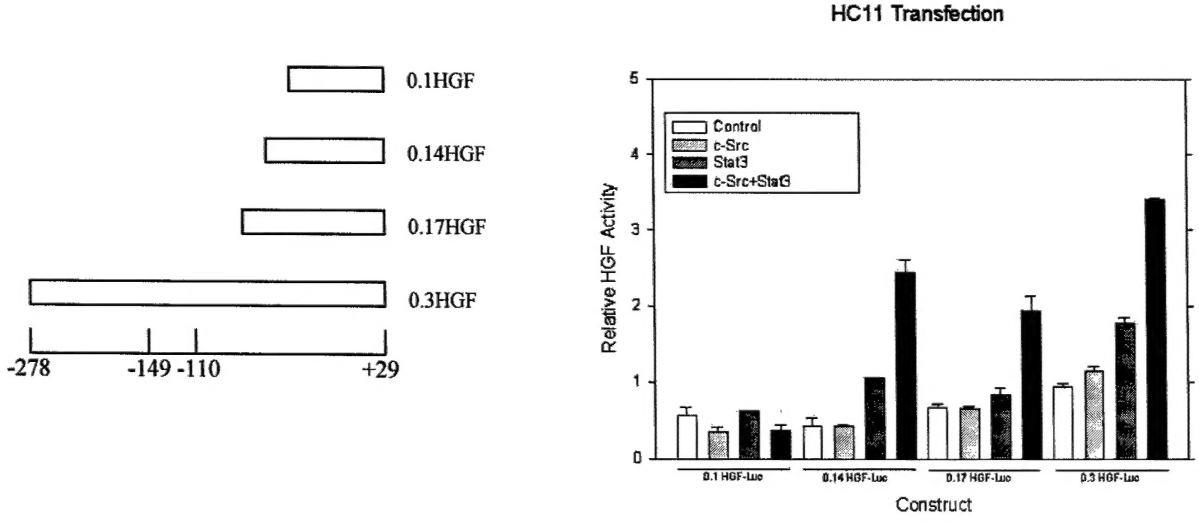
Progress and Results

1. Identification of the site responsible for c-Src/Stat3 responsiveness of HGF promoter.

The c-Src/Stat3 responsive region, contained between –254 and –70 base pairs of the *HGF* promoter, will be studied using a mutational approach. Two sites (-149 and –110) were originally identified as putative consensus Stat3 binding sites, and mutants for both sites were constructed. Additionally a double mutant (with both sites mutated) was developed. The Luciferase assay results failed to show an inhibition of responsiveness to c-Src/Stat3 in those mutants. (Fig.1)



A third candidate binding site for Stat3 was identified at –95 position. An initial experiment using a new deletion mutant (which contains –95 but not –149 and –110) shows that this site is sufficient for induction of *HGF* promoter responsiveness to c-Src/Stat3. (Fig.2)



Based on this result the construction of a new mutant of the HGF promoter, lacking the newly identified Stat3 binding sequence, is planned, and will show if the –95 site is necessary for *HGF* responsiveness to c-Src/Stat3 induction.

To confirm that upstream regulatory regions of the *HGF* promoter constructs are functionally intact, estrogen responsiveness, which maps at position –872 bp will be assessed, using fibroblasts as host cells.

2. Testing of activated Stat3 function in HGF expression

The responsiveness of *HGF* to Stat3 that has been observed to this point is conditioned by the presence of activated Src (Figs. 1&2). To further explore the role of Stat3 in *HGF* regulation, HC11 cells will be transfected with an activated Stat3 construct, and its capacity of regulating *HGF* transcription will be tested.

To confirm that the changes in promoter activity translate into changes in HGF protein levels, those will be assessed in HC11 cells stably transfected with activated c-Src, Stat3 or activated c-Src/Stat3.

3. Characterization of DNA-protein complex formation

Supershift studies with antibodies against specific Stat proteins identified Stat3, and excluded Stat1, -5A and –5B, as a component of the DNA-protein complex. The two sites originally identified as candidate Stat3 binding sequences (-149 and –110) were used as probes, and proved to form DNA-protein complexes when nuclear extracts were added. Band shifting experiments will be performed using the newly identified candidate Stat3 binding sequence.

Key Research Accomplishments

- Introduction of point and deletion mutations into the HGF promoter.
- Identification of a new putative Stat3 binding site on the HGF promoter.

Reportable Outcomes

- Dr. Ewa Joanna Wojcik was appointed as a new trainee supported by this award (July 1, 2002 to June 30, 2003)
- Abstract and poster presentation: Elliott B, Tremblay E, Wojcik J, Hung W. src and Stat3 regulate HGF expression and epithelial mesenchymal transition in mammary carcinoma cells. Presented at the Era of Hope Meeting, Orlando, Florida, September 2002

Conclusions

This study will identify signalling molecules that regulate *HGF* promoter activity preferentially in carcinoma cells, which could define a potentially new level of tumor specificity that might be associated with aberrant HGF expression in breast cancer. This information could lead to novel strategies for design of small molecule antagonists, such as derivative peptides or decoy oligos, to inhibit *HGF* gene expression in tumor cells with minimal effects on normal HGF/Met function.

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SRC AND STAT3 REGULATE HGF EXPRESSION AND EPITHELIAL MESENCHYMAL TRANSITION IN MAMMARY CARCINOMA CELLS

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We have previously shown coexpression of hepatocyte growth factor (HGF) and its receptor Met in the invasive tumor front of human breast carcinomas. We have also demonstrated secretion of HGF, constitutive activation of Met, and increased invasion in a murine breast carcinoma cell line, SP1. These observations suggest the presence of an HGF autocrine loop in some breast carcinoma cells, which confers increased survival, growth, and invasiveness during tumor progression and metastasis. c-Src tyrosine kinase, which is critical in regulating the expression of many genes, is activated in SP1 carcinoma cells, as well as in most human breast cancers. We therefore examined the role of c-Src kinase in HGF expression in breast carcinoma cells.

Our approach was to use both pharmacological and mutational approaches to modulate c-Src activity in carcinoma cells. Expression of an activated c-Src mutant in SP1 cells increased transcription from the *HGF* promoter and expression of HGF mRNA and protein, while dominant negative c-Src had the opposite effect. Using deletion analysis, we showed that the region between -254 and -70 base pairs was required for c-Src responsiveness of the *HGF* promoter. This region contains two putative consensus sequences (at -110 and -149 bps) for the Stat3 transcription factor, which bind protein complexes containing Stat3 (but not Stat1, -5A, or -5B). This region is distinct from the epithelial-specific negative regulatory site (-16 to + 4 bp), the TGF-β inhibitory element (-364 to –355 bp), or sites involved in estrogen responsiveness (-872 to –860 bp).

Coexpression of activated c-Src and Stat3 synergistically induced strong *HGF* promoter activity in SP1 carcinoma cells. c-Src kinase activity correspondingly increased the tyrosine 705 phosphorylation and DNA binding affinity of Stat3 (but not Stat1, -5A, or -5B). In addition, over-expression and activation of c-Src and Stat3 in mammary epithelial cells causes strong *HGF* promoter activity and secretion of active HGF, concomitant with marked cell scattering and migration. Collectively, our data indicate a cooperative effect of c-Src kinase and Stat3 in the activation of *HGF* transcription and protein expression in breast carcinoma cells. This process may be important in overriding the strong repression of HGF expression in nonmalignant epithelium, and thereby promote tumorigenesis.

We are currently examining whether targeting Stat3 signaling with peptidomimetic and oligonucleotide inhibitors can block autocrine HGF expression, Met activation, and invasion in mammary carcinoma cells.

Information from this study could lead to novel approaches for therapeutic intervention in breast cancer metastasis.

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